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86.73918

2. Patent application number
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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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Patents ADP number *(if you know it)* 7542335001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

Method for identifying substances which positively influence inflammatory conditions of chronic inflammatory airway diseases.

5. Name of your agent *(if you have one)*

Frank B. Dehn & Co

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Method For Identifying Substances Which Positively Influence Inflammatory Conditions Of Chronic Inflammatory Airway Diseases

Introduction

The present invention belongs to the field of modulation of inflammatory processes, in particular of inflammatory airway diseases, in which macrophages play an important role. The inflammatory processes can be modulated according to the invention by influencing the function of receptors on macrophages, which receptors are identified to be involved in the inflammatory process.

Inflammatory processes involve a cascade of reactions. A wide variety of factors are involved in inflammatory processes leaving a single treatment to avoid said factors unsuccessful. This is in particular true for inflammatory processes of the airways, like the chronic inflammatory airway diseases.

Chronic inflammatory airway diseases include Chronic Bronchitis and Chronic Obstructive Pulmonary Disease (COPD). For example, COPD is a complex disease encompassing symptoms of several disorders: chronic bronchitis which is characterized by cough and mucus hypersecretion, small airway disease, including inflammation and peribronchial fibrosis, and emphysema. COPD is characterized by an accelerated and irreversible decline of lung function. The major risk factor for developing COPD is continuous cigarette smoking. Since only about 20% of all smokers are inflicted with COPD, a genetic predisposition is also likely to contribute to the disease.

The initial events in the early onset of COPD are inflammatory, affecting small and large airways. An irritation caused by cigarette smoking attracts macrophages and neutrophils the number of which is increased in the sputum of smokers. Perpetual smoking leads to an ongoing inflammatory response in the lung by releasing mediators from macrophages, neutrophils and epithelial cells that recruit inflammatory cells to sites of the injury. So far there is no therapy available to reverse the course of COPD. Smoking cessation may reduce the decline of lung function. Only a few drugs provide some relief for patients. Longlasting β2-agonists and anticholinergics are applied to achieve a transient bronchodilatation. A variety of antagonists for inflammatory events are under investigation like, LTB_4-, IL-8-, TNFα-inhibitors.

Chronic inflammatory airway diseases can be attributed to activated inflammatory immune cells, e.g. macrophages. There is a need for modulating the function of macrophages in order to eliminate a basis for inflammatory processes.

Description Of The Invention

In the present invention it was found that macrophages involved in an inflammatory process, preferably in a chronic inflammatory airway disease, more preferably in chronic bronchitis or COPD, show a pattern of differentially expressed nucleic acid sequence and protein expression which differs from the pattern of gene expression of macrophages from healthy donors or donors in an irritated status, which latter do contain macrophages in an activated status. Therefore, macrophages show different activation levels under different inflammatory conditions and it is shown in the present invention that macrophages in an hyperactive status are involved in an inflammatory process, preferably in a chronic inflammatory airway disease, more preferably in chronic bronchitis or COPD. The present invention provides for the inhibition of the hyperactivation or the reduction of the hyperactive status of a macrophage by allowing the identification of substances which modulate receptors involved in the hyperactivation or maintaining the hyperactive status.

The invention is based on the identification of a differentially expressed nucleic acid sequence or protein which is involved in causing the induction and/or maintenance of the hyperactive status of macrophages involved in an inflammatory process, preferably in a chronic inflammatory airway disease, more preferably in chronic bronchitis or COPD. Such differentially expressed nucleic acid sequence or protein is in the following named differentially expressed nucleic acid sequence or protein of the invention respectively. In particular, the present invention teaches a link between phenotypic changes in macrophages due to differentially expressed nucleic acid sequence and protein expression pattern and involvement of macrophages in inflammatory processes and, thus, provides a basis for a variety of applications. For example, the present invention provides a method and a test system for determining the expression level of a macrophage protein or differentially expressed nucleic acid sequence of the invention and thereby provides e.g. for methods for diagnosis or monitoring of inflammatory processes with involvement of hyperactivated macrophages in mammalian, preferably human beings, especially such beings suffering from an inflammatory process, preferably in a chronic inflammatory airway disease, more preferably in chronic bronchitis or COPD. The invention also relates to a method for identifying a substance by means of a differentially expressed nucleic acid sequence or protein of the invention processes, which substance modulates, i.e. acts as an inhibitor or activator on the said differentially expressed

nucleic acid sequence or protein of the invention and thereby positively influences chronic inflammatory processes by inhibition of the hyperactivation or reduction of the hyperactive status of macrophages, and thereby allows treatment of mammals, preferably human beings, suffering from a said disease. The invention also relates to a method for selectively modulating such a differentially expressed nucleic acid sequence or protein of the invention in a macrophage comprising administering a substance determined to be a modulator of said protein or differentially expressed nucleic acid sequence. The present invention includes the use of said substances for treating beings in need of a treatment of an inflammatory process, preferably a chronic inflammatory airway disease, more preferably chronic bronchitis or COPD.

For the present invention in a first step differentially expressed nucleic acid sequences and proteins are identified which have a different expression pattern in a hyperactivated macrophage compared to a macrophage which is not hyperactivated. For the sake of conciseness this description deals particularly with investigation of macrophages involved in COPD, however, equivalent results may be observed with samples from patients suffering from other chronic inflammatory airway diseases, e.g. chronic bronchitis. The investigation of the different expression pattern leads to the identification of a series of differentially expressed nucleic acid sequences in macrophages, differentially expressed in dependency on the activation status of a macrophage involved in an inflammatory process, as exemplified in the Examples hereinbelow.

Briefly, such a differentially expressed differentially expressed nucleic acid sequence is identified by comparative expression profiling experiments using a cell or cellular extract from a hyperactivated macrophage, i.e. for example from the site of inflammation in a COPD and from the corresponding site of control being not suffering from said disease, however, suffering from an irritated condition like cigarette smoke exposure.

A differentially expressed nucleic acid sequence or protein of the invention can easily be detected by such a method because amongst the differentially expressed macrophage genes a class of differentially expressed nucleic acid sequences can be identified which encodes a class of macrophage surface receptors which is characterized in that it is expressed at a lower or higher level than the control level in a macrophage which is not hyperactivated. Such a macrophage surface receptor of the invention is hereinafter named ILM receptor. However, the invention does not only concern a naturally occurring ILM receptor, but also includes within the meaning

of ILM receptor a receptor which is functionally equivalent to, i.e. which shares the binding capacities and the cellular function with an ILM receptor.

An example for an ILM receptor according to the present invention is a FPRL-1 receptor type receptor including FPRL-1 receptor (SEQ ID NO. 2). The term "receptor type receptor" used in context with the present invention, e.g. FPRL-1 receptor type receptor, is a receptor which is "functionally equivalent" to, i.e. which shares the binding capacities and the cellular function with, the respective receptor, e.g. FPRL-1 receptor of Seq. ID NO. 2; the term also encompasses variants, mutants or fragments of a naturally occuring receptor, e.g. FPRL-1 receptor. or naturally occuring receptor type receptor, e.g. FPRL-1 receptor type receptor, which variants, mutants or fragments are functionally equivalent to the receptor, e.g. FPRL-1 receptor.

Further examples for ILM receptors are HM74 receptor type receptor including HM74 receptor (SEQ ID NO. 21); AICL receptor type receptor including AICL receptor (SEQ ID NO. 6)); ILT1 receptor type receptor including ILT1 receptor (SEQ ID NO. 12); SHPS-1 receptor type receptor including SHPS-1 receptor (SEQ ID NO. 4); KDEL receptor 1 type receptor including KDEL receptor 1 (SEQ ID NO. 8); and CSF-1 receptor type receptor including CSF-1 receptor (SEQ ID NO. 10). Preferred is the respective receptor shown in the sequence listing or a variant, mutant or fragment thereof having the same function, even more preferred is the respective receptor shown in the sequence listing under SEQ ID NO. 21, 6, 12, 4, 8, 10. In even more preferred embodiments the receptors are encoded by the nucleic acid sequences having the SEQ ID NOs 20, 5, 11, 3, 7 or 9, respectively.

A preferred embodiment of an ILM receptor in context with the present invention is a FPRL-1 receptor type receptor. The term FPRL-1 receptor type receptor accordingly also encompasses variants, mutants or fragments, of naturally occuring FPRL-1 receptor or FPRL-1 receptor type receptors, which variants, mutants or fragments are functionally equivalent to the FPRL-1 receptor. An even more preferred embodiment in context with the description of the embodiments of the present invention is the FPRL-1 receptor of Seq. ID NO. 2 or a variant, mutant or fragment thereof having the same function, even more preferred is the FPRL-1 receptor of Seq. ID NO 2. In a most preferred embodiment, the FPRL-1 receptor is encoded by the nucleic acid sequence shown in SEQ ID NO. 1.

According to the present invention, the function of an ILM receptor expressed at a lower level than the control level is preferably activated in order to inhibit

hyperactivation or reduce a hyperactivated status of a macrophage, whereby the function of an ILM receptor which is expressed at a higher level than the control level is preferably inhibited in order to inhibit hyperactivation or reduce a hyperactivated status of a macrophage. Function of a receptor in context with the present invention is any function of a receptor of the invention which is capable of influencing the inflammatory processes. For example, a receptor of the invention is mediating inflammation in that it is activated by a ligand (every substance which has the capacity to bind to said receptor at least one of its domains exposed on the cell surface) and leads to a intracellular signal involved in inflammatory processes.

In one embodiment the present invention concerns a method for determining a substance to be an activator or inhibitor of an ILM receptor characterized in that the receptor is overexpressed in a macrophage involved in a chronic inflammatory airway disease and which receptor plays a role in mediating inflammation. A test system useful for performing such method of the invention comprises a cell or a cell-free system. For example, in one embodiment according to the invention the system is designed in order to allow the testing of substances acting on the expression level of the differentially expressed nucleic acid sequence, in another embodiment the system allows the testing of substances directly interacting with the receptor or interfering with the binding of the receptor with a natural or an artificial but appropriate ligand. The latter system comprises a receptor of the invention in a way that a substance which should be tested can physically contact said receptor and which direct interaction leads to a measurable read-out indicative for the change of receptor function.

The present invention also provides a test system for determining whether a substance is an activator or an inhibitor according to the invention of an ILM receptor function according to the invention. For performing a method for determining whether a substance is an activator or an inhibitor of receptor function of the present invention cells as well as cell-free systems can be used. Test systems for performing the method can be, for example, designed and built up by using elements and methods well known in the art. For example, cell-free systems may include, for example, cellular compartments or vesicles comprising a receptor of the invention. Suitable cellular systems include, for example, a suitable prokaryotic cell or eukaryotic cell, i.e. such comprising a respective receptor of the invention. A cell suitable for performing a said method of the invention may be obtained by recombinant techniques, i.e. after transformation or transfection with a vector suitable for expression of the desired receptor of the invention, or may be a cell line or a cell isolated from a natural source expressing the desired receptor of the

invention. The test system may include a natural or artificial ligand of the receptor if desirable or necessary for testing whether a substance of interest is an inhibitor or activator of a receptor of the invention.

A test method according to the invention comprises measuring a read-out, i.e. a phenotypic change in the test system, for example, if a cellular system is used a phenotypic change of the cell. Such change may be a change in a naturally occurring or artificial response of the cell to receptor activation or inhibition, e.g. as detailed in the Examples hereinbelow.

A test method according to the invention can on the one hand be useful for high throughput testing suitable for determining whether a substance is an inhibitor or activator of the invention, but also e.g. for secondary testing or validation of a hit or lead substance identified in high throughput testing.

The present invention also concerns a substance identified in a method according to the invention to be an inhibitor or activator of a receptor of the invention. A substance of the present invention is any compound which is capable of activating or preferably inhibiting a function of a receptor according the invention. An example of a way to activate or inhibit a function of a receptor is by influencing the expression level of said receptor. Another example of a way to activate or inhibit a function of a receptor is to apply a substance directly binding the receptor and thereby activating or blocking functional domains of said receptor, which can be done reversibly or irreversibly, depending on the nature of the substance applied.

Accordingly, a substance useful for activating or inhibiting receptor function include substances acting on the expression of differentially expressed nucleic acid sequence, but also such acting on the receptor itself. Therefore, according to the invention the meaning of the term a substance of the invention includes but is not limited to nucleic acid sequences coding for the gene of a receptor of the invention or a fragment or variant thereof and being capable of influencing the gene expression level, e.g. nucleic acid molecules suitable as antisense nucleic acid, ribozyme, or for triple helix formation. Another substance of the invention is e.g. an antibody or an organic or inorganic compound directly binding to or interfering with the binding of an appropriate ligand with a receptor of the invention and thereby affecting its function.

In a further aspect, the present invention relates to a method for determining an expression level of an ILM receptor differentially expressed nucleic acid sequence or

protein according to the invention comprising determining the level of said ILM receptor in a macrophage according to the invention. Such a method can be used, for example, for testing whether a substance is capable of influencing differentially expressed nucleic acid sequence expression levels in a method outlined above for determining whether a substance is an activator or inhibitor. A method for determining an expression level of an ILM receptor differentially expressed nucleic acid sequence or protein can, however, also be used for testing the activation status of a macrophage, e.g. for diagnostic purposes or for investigation of the success of treatment of a disease which is caused by the hyperactivated macrophage. Accordingly, the invention also relates to a method for diagnosis of a chronic inflammatory disease or monitoring of such disease, e.g. monitoring success in treating beings in need of treatment of such disease, comprising determining the level of the receptor expressed in a macrophage according to the invention. Said macrophage is preferably a mammalian, more preferably a human cell. Accordingly, macrophages of the present invention are preferably obtainable from the site of inflammation in a mammal and more preferably from a site of inflammation in a human being.

A method for determining expression levels of a receptor according to the invention can depending on the purpose of determining the expression level be performed by known procedures such as measuring the concentration of respective RNA transcripts via hybridization techniques or via reporter gene driven assays such as luciferase assays or by measuring the protein concentration of said receptor using respective antibodies to verify the identity of said protein.

The present invention relates to the use of a substance according to the invention for the treatment of a chronic inflammatory airways disease according to the invention. Another embodiment of the present invention relates to a pharmaceutical composition comprising at least one of the substances according to the invention determined to be an activator or an inhibitor using the method for determining whether the substance is an activator or an inhibitor according to the invention characterized in that the respective receptor according to the invention is overexpressed in a macrophage according to the invention involved in a chronic inflammatory airway disease according to the invention. The composition may be manufactured in a manner that is itself known, e.g. by means of conventional mixing, dissolving, granulating, dragee-making, levigating, powdering, emulsifying, encapsulating, entrapping or lyophilizing processes.

In order to use substances activating or inhibiting according to the invention as drugs for treatment of chronic inflammatory airway diseases, the substances can be tested in animal models for example an animal suffering form an inflammatory airway disorder or a transgenic animal expressing a receptor according to the invention. Toxicity and therapeutic efficacy of a substance according to the invention can be determined by standard pharmaceutical procedures, which include conducting cell culture and animal experiments to determine the IC_{50}, LD_{50} and ED_{50}. The data obtained are used for determining the animal or more preferred the human dose range, which will also depend on the dosage form (tablets, capsules, aerosol sprays ampules, etc.) and the administration route (for example transdermal, oral, buccal, nasal, enteral, parenteral, inhalative, intratracheal, or rectal).

A pharmaceutical composition containing a least one substance according to the invention as an active ingredient can be formulated in conventional manner. Methods for making such formulations can be found in manuals, e.g. "Remington Pharmaceutical Science". Examples for ingredients that are useful for formulating at least one substance according to the present invention are also found in WO 99/18193, which is hereby incorporated by reference.

In a further aspect the invention teaches a method for treating a chronic inflammatory airway disease according to the invention which method comprises administering to a being preferably to a human being in need of such treatment a suitable amount of a pharmaceutical composition comprising at least one substance determined to be an activator or inhibitor according to a method for determining whether a substance is an activator or an inhibitor according to the invention of an ILM receptor according to the invention characterized in that the receptor is overexpressed in a macrophage according to the invention and plays a role in mediating inflammation involved in a chronic inflammatory airway disease according to the invention.

In an other embodiment the invention relates to a method for selectively modulating ILM receptor concentration in a macrophage, comprising administering a substance determined to be an activator or inhibitor of a receptor according to the invention.

The following examples are meant to illustrate the present invention, however, shall not be construed as limitation. However, the Examples describe most preferred embodiments of the invention.

Examples

Example 1: Comparative Expression Profiling and FPLR-1 Cloning

The following is an illustration of how comparative expression profiling can be performed in order to identify receptors according to the present invention.

1.1. Selection of Patients

Three groups of subjects are studied: healthy non-smokers, healthy smokers and patients with COPD.

In order to assess lung function subjects have to perform spirometry. A simple calculation based on age and height is used to characterise the results. COPD subjects are included if their FEV_1 % predicted is <70%. Healthy smokers are age and smoking history matched with the COPD subjects but have normal lung function. Healthy non-smokers have normal lung function and have never smoked. The latter group has a methacholine challenge to exclude asthma. This technique requires increasing doses of methacholine to be given to the subject, with spirometry between each dose. When the FEV_1 falls 20% the test is stopped and the PC_{20} is calculated. This is the dose of methacholine causing a 20% fall in FEV_1 and we will require a value of >32 as evidence of absence of asthma. All subjects have skin prick tests to common allergens and are required to have negative results. This excludes atopic individuals. The clinical history of the subjects is monitored and examined in order to exclude concomitant disease.

1.2. BAL (bronchoalveolar lavage) Procedure

Subjects are sedated with midazolam prior to the BAL. Local anaesthetic spray is used to anaesthetize the back of the throat. A 7mm Olympus bronchoscope is used. The lavaged area is the right middle lobe. 250 ml of sterile saline is instilled and immediately aspirated. The resulting aspirate contains macrophages.

1.3. BAL Processing

BAL is filtered through sterile gauze to remove debris. The cells are washed twice in HBSS, resuspended in 1ml HBSS (Hank's Balanced Salt Solution) and counted. The macrophages are spun to a pellet using 15 ml Falcon blue-cap polypropylen, resuspended in Trizol reagent (Gibco BRL Life Technologies) at a concentration of 1 ml Trizol reagent per 10 million cells and then frozen at -70°C.

1.4. Differential Gene Expression Analysis

Total RNA is extracted from macrophage samples obtained according to Example 1.3. Cell suspensions in Trizol are homogenized through pipetting and incubated at room temperature for 5 minutes. 200 µl chloroform per ml Trizol is added, the mixture carefully mixed for 15 seconds and incubated for 3 more minutes at room temperature. The samples are spun at 10000g for 15 minutes at 4°C. The upper phase is transferred into a new reaction tube and the RNA is precipitated by adding 0.5 ml isopropanol per ml Trizol for 10 minutes at room temperature. Then, the precipitate is pelleted by using a microcentifuge for 10 minutes at 4°C with 10000g, the pellet is washed twice with 75% ethanol, air dried and resuspended in DEPC-H_2O.

An RNA cleanup with Qiagen RNeasy Total RNA isolation kit (Qiagen) is performed in order to improve the purity of the RNA. The purity of the RNA is determined by agarose gelelectrophoresis and the concentration is measured by UV absorption at 260 nm.

5 µg of each RNA is used for cDNA synthesis. First and second strand synthesis are performed with the SuperScript Choice system (Gibco BRL Life Technologies). In a total volume of 11 µl RNA and 1 µl of 100 µM T7-$(dt)_{24}$ primer, sequence shown in SEQ ID NO. 13, are heated up to 70°C for 10 minutes and then cooled down on ice for 2 minutes. First strand buffer to a final concentration of 1x, DTT to a concentration of 10 mM and a dNTP mix to a final concentration of 0.5 mM are added to a total volume of 18 µl. The reaction mix is incubated at 42°C for 2 minutes and 2 µl of Superscript II reverse transcriptase (200 U/µl) are added. For second strand synthesis 130 µl of a mix containing 1.15x second strand buffer, 230 µM dNTPs, 10 U E.coli DNA ligase (10U/µl), E.coli DNA polymerase (10 U/µl), RNase H (2U/µl) is added to the reaction of the first strand synthesis and carefully mixed with a pipette. Second strand synthesis is performed at 16°C for 2 hours, then 2 µl of T4 DNA polymerase (5 U/µl) are added, incubated for 5 minutes at 16°C and the reaction is stopped by adding 10 µl 0,5 M EDTA.

Prior to cRNA synthesis the double stranded cDNA is purified. The cDNA is mixed with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) and spun through the gel matrix of phase lock gels (Eppendorf) in a microcentrifuge in order to separate the cDNA from unbound nucleotides. The aqueous phase is precipitated with ammoniumacetate and ethanol. Subsequently, the cDNA is used for in vitro transcription. cRNA synthesis is performed with the ENZO BioArray High Yield RNA Transcript Labeling Kit according to manufacturer's protocol (ENZO Diagnostics). Briefly, the cDNA is incubated with 1x HY reaction buffer, 1x biotin labeled ribonucleotides, 1x DTT, 1x RNase Inhibitor Mix and 1x T7 RNA Polymerase in a

total volume of 40 µl for 5 hours at 37°C. Then, the reaction mix is purified via RNeasy columns (Qiagen), the cRNA precipitated with ammonium acetate and ethanol and finally resuspended in DEPC-treated water. The concentration is determined via UV spectrometry at 260 nm. The remaining cRNA is incubated with 1x fragmentation buffer (5x fragmentation buffer: 200 mM Tris acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc) at 94°C for 35 minutes.

For hybridization of the DNA chip 15 µg of cRNA is used, mixed with 50 pM biotin-labeled control B2 oligonucleotide, sequence shown SEQ ID NO. 14, 1x cRNA cocktail, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, 1x MES (2-[N-morpholino]-ethanesulfonic acid) hybridization buffer in a total volume of 300 µl. The hybridization mixture is heated up to 99°C for 5 minutes, cooled down to 45°C for 10 minutes and 200 µl of the mix are used to fill the probe array. The hybridization is performed at 45°C at 60 rpm for 16 hours.

After the hybridization the hybridization mix on the chip is replaced by 300 µl non-stringent wash buffer (100 mM MES, 100 mM NaCl, 0.01% Tween 20). The chip is inserted into an Affymetrix Fluidics station and washing and staining is performed according to the EukGE-WS2 protocol. The staining solution per chip consists of 600 µl 1x stain buffer (100 mM MES, 1 M NaCl, 0.05% Tween 20), 2 mg/ml BSA, 10 µg/ml SAPE (streptavidin phycoerythrin) (Dianova), the antibody solution consists of 1x stain buffer, 2 mg/ml BSA, 0.1 mg/ml goat IgG, 3 µg/ml biotinylated antibody. After the washing and staining procedure the chips are scanned on the HP Gene Array Scanner (Hewlett Packard).

Data Analysis is performed by pairwise comparisons between chips hybridized with RNA isolated from COPD smokers and chips hybridized with RNA isolated from healthy smokers.

One of the different expressed nucleic acid sequences identified is coding for FPRL-1 (formyl peptide receptor like-1) receptor (also named LXA_4R, HM63, FPR2, FPRH2, FMLP-R-II, Lipoxin A4 receptor); see Seq ID NOs. 1 and 2. It belongs to the chemoattractant peptide receptor family including receptors for fMLP (N-formyl-methionyl-leucyl-phenylalanine), IL-8 or C5a. These receptors show a seven-transmembrane helix motif and signal through heterotrimeric G-proteins. FPRL-1 receptor was identified as the high-affinity receptor for lipoxin A_4 (LXA_4) (Murphy et al. 1992).

Alveolar macrophages have been shown to produce lipoxins, which are synthesized by 15-lipoxygenase (Kim 1988). Lipoxin A_4 (LXA_4) stimulates chemotaxis, adherence and calcium release in monocytes. In neutrophils, though, LXA_4 inhibits chemotaxis and adhesion, and downregulates transmigration through epithelial cells (Maddox

and Serhan 1996). LXA_4 was found elevated in BALs from patients with asthma (Lee et al. 1990, Serhan 1999). In particular, it was found to cause a dose-dependent contraction of human bronchi (Christie et al. 1992). LXA_4 is considered to be a generic modulator of inflammation in the lung.

1.5. FPRL-1 receptor Overexpressed in COPD Macrophages

FPRL-1 receptor is consistently found upregulated (66.7%) in COPD smokers compared to healthy smokers. This is demonstrated by calculated "fold change" values from 42 pairwise comparisons and by average difference ("avg diff") values (Table 1, 2). Relative expression levels for non-smokers and healthy smokers are similar and elevated levels are restricted to patients with COPD. Therefore, COPD-specific effects cause the upregulation.

Table 1: Expression pattern for FPRL-1 receptor: fold change calculation for 42 pairwise comparisons between COPD and healthy smokers. Only values higher than 2fold and lower than –2fold are considered as deregulated. Thus FPRL-1 receptor was 28 times upregulated and 14 times not regulated.

fold change	comparison	fold change	comparison	fold change	comparis
2.7	39vs2	2.9	5vs2	3.3	1vs2
4.6	39vs37	3.6	5vs37	5.5	1vs37
2	39vs43	1.4	5vs43	1.4	1vs43
3.1	39vs56	3	5vs56	3.9	1vs56
4.1	39vs57	3.2	5vs57	5.3	1vs57
2.9	39vs58	3	5vs58	3.6	1vs58
2.2	39vs62	2.7	5vs62	2.7	1vs62
1.3	44vs2	2.7	6vs2	1.4	3vs2
2.7	44vs37	4.1	6vs37	2.9	3vs37
-1.9	44vs43	1.1	6vs43	-1.7	3vs43
1.5	44vs56	3.2	6vs56	1.7	3vs56
2	44vs57	3.5	6vs57	2.3	3vs57
1.4	44vs58	2.9	6vs58	1.5	3vs58
1.1	44vs62	2.2	6vs62	1.2	3vs62

Table 2: Expression levels of FPRL-1 receptor: "avg diff" values, a relative indicator of the intensity of the hybridisation signal on the chip, for each patient are listed; OS means obstructed smoker, HS healthy smoker, NS non-smoker

OS	avg diff	HS	avg diff	NS	avg diff
P 1	1276.7	P 2	490.4	P 48/49	248.2
P 3	553.6	P 37	52.1	P 50/52	565.7
P 5	1710.2	P 43	940	P 54/61	142.4
P 6	1046.9	P 56	327.1		
P 39	1025.2	P 57	238.7		
P 44	507.1	P 58	358.2		
		P62	469.6		
mean + std. dev.		1020.0 ± 452.5		410.9 ± 276.3		318.8 ± 220.3
median		1036.1		327.1		248.2

P value for comparisons between COPD smokers and healthy smokers: 0.02

Chip data for FPRL-1 receptor are confirmed by TaqMan analysis (Perkin Elmer Applied Biosystems) for three COPD and two healthy smokers. Fold changes obtained by TaqMan very much resemble the data from the gene chips (Table 3).

Table 3: Upregulation of FPRL-1 receptor in COPD smokers determined by gene chips and TaqMan.

Fold change determination for FPRL-1 receptor by chip data in six comparisons between COPD smokers and healthy smokers is validated by analysis of the same samples by TaqMan and the relative upregulation is calculated with GAPDH as a housekeeping gene.

comparison	chip	TaqMan
1vs2	3.3	4.1
3vs2	1.4	2.2
39vs2	2.7	6.0

comparison	chip	TaqMan
1vs37	5.5	4.6
3vs37	2.9	2.5
39vs37	4.6	6.8

Another differentially expressed nucleic acid sequence identified codes for HM74 receptor, see SEQ ID NOs. 20 and 21, which belongs to the family of G-protein-coupled receptors. HM74 receptor was cloned from a human monocytic library (Nomura et al. 1993). To date, the ligand has not been identified. HM74 receptor is consistently found upregulated (54.8%) in COPD smokers compared to healthy smokers. This is demonstrated by calculated „fold change" values (Table 5) from 42 pairwise comparisons and by „avg diff" values (Table 6).

Table 5. Expression pattern for HM74 receptor: fold change calculation for 42 pairwise comparisons between COPD and healthy smokers. Only values higher than 2fold and lower than –2fold are considered as deregulated. Thus, HM74 receptor was 23 times upregulated and 17 times not regulated

fold change	comparison	fold change	comparison	fold change	comparis.
1.2	39vs2	4.5	5vs2	-1.2	1vs2
4.7	39vs37	13.8	5vs37	2.8	1vs37
-2.1	39vs43	2.5	5vs43	-2.2	1vs43
2.9	39vs56	8.6	5vs56	1.8	1vs56
2.6	39vs57	8.9	5vs57	1.6	1vs57
2.6	39vs58	7.7	5vs58	1.6	1vs58
2.4	39vs62	8.5	5vs62	1.5	1vs62
2.8	44vs2	1	6vs2	-1.1	3vs2
8.8	44vs37	3.5	6vs37	3	3vs37
1.5	44vs43	-1.7	6vs43	-2	3vs43
5.5	44vs56	2.2	6vs56	1.9	3vs56
5.4	44vs57	2	6vs57	1.7	3vs57
4.9	44vs58	1.9	6vs58	1.7	3vs58
5.2	44vs62	1.9	6vs62	1.7	3vs62

Table 6: Expression levels of HM74 receptor: "avg diff" values, a relative indicator of the intensity of the hybridisation signal on the chip, for each patient are listed; OS means obstructed smoker, HS healthy smoker, NS non-smoker

OS	avg diff	HS	avg diff	NS	avg diff
P 1	3233	P 2	3916.3	P 48/49	1690.7
P 3	3474.5	P 37	1154.5	P 50/52	4176.4
P 5	17671	P 43	5770.5	P 54/61	3504.8
P 6	4094.2	P 56	1860.2		
P 39	4201.3	P 57	1639.8		
P 44	11068.5	P 58	2080.2		
		P62	1721.6		
mean + std. dev.		7290.4 ± 5879.0		2591.9 ± 1652.5		3124.0 ± 1285.9
median		4147.8		2243.6		3504.8

Chip data for HM74 receptor are confirmed by TaqMan analysis for three COPD and two healthy smokers. Fold changes obtained by TaqMan very much resemble the data from the gene chips (Table 7).

Table 7: Upregulation of HM74 receptor in COPD smokers determined by gene chips and TaqMan.

Fold change determination for HM74 receptor by chip data in six comparisons between COPD smokers and healthy smokers is validated by analysis of the same samples by TaqMan and the relative upregulation is calculated with GAPDH as a housekeeping gene.

comparison	chip	TaqMan
1vs2	0.8	2.3
3vs2	0.9	0.8
39vs2	1.2	1.4

comparison	chip	TaqMan
1vs37	2.8	4.5
3vs37	3.0	1.4
39vs37	4.7	2.6

Another differentially expressed nucleic acid sequence identified codes for AICL receptor (activation-induced C-type lectin), see SEQ ID NOs. 5 and 6., which is a type II membrane protein that recognizes and binds N-acetyl-galactosamin or –glucosamin moieties of plasma glycoproteins (Oda et al. 1988). It is expressed in lymphoid tissues and in hematopoetic cells as well as in NK and T cells. Its expression is induced during lymphocyte activation and after stimulation with PMA (Hamann et al. 1997). Since homologues of AICL receptor are involved in signal transmission in lymphocytes and in lymphocyte proliferation, it is tempting to assume that AICL receptor also participates in these processes (Hamann et al. 1993). AICL receptor is consistently found upregulated (66.7%) in COPD smokers compared to healthy smokers. This is demonstrated by calculated „fold change" values (Table 8) from 42 pairwise comparisons and by „avg diff" values (Table 9). The p value for the comparisons between COPD smokers and healthy smokers was 0.01.

Table 8. Expression pattern for AICL receptor: fold change calculation for 42 pairwise comparisons between COPD and healthy smokers. Only values higher than 2fold and lower than –2fold are considered as deregulated. Thus, AICL receptor was 28 times upregulated and 14 times not regulated

fold change	comparison	fold change	comparison	fold change	comparis.
1	39vs2	1.5	5vs2	-1.3	1vs2
1.9	39vs37	2.8	5vs37	1.4	1vs37
-1.4	39vs43	2.4	5vs43	1.3	1vs43
3.3	39vs56	5	5vs56	2.7	1vs56
6.9	39vs57	10	5vs57	5.3	1vs57
3.1	39vs58	4.5	5vs58	2.3	1vs58
3.3	39vs62	5.1	5vs62	2.7	1vs62
1.4	44vs2	-1.4	6vs2	-1.5	3vs2
2.6	44vs37	1.2	6vs37	1.2	3vs37
2.3	44vs43	1.1	6vs43	1.1	3vs43
4.2	44vs56	2.3	6vs56	2.3	3vs56
9.6	44vs57	4.5	6vs57	4.5	3vs57
4.3	44vs58	2	6vs58	2	3vs58
4.2	44vs62	2.3	6vs62	2.3	3vs62

Table 9: Expression levels of AICL receptor: "avg diff" values, a relative indicator of the intensity of the hybridisation signal on the chip, for each patient are listed; OS means obstructed smoker, HS healthy smoker, NS non-smoker

OS	avg diff	HS	avg diff	NS	avg diff
P 1	3415.3	P 2	4984.2	P 48/49	748.4
P 3	3412.9	P 37	2388.6	P 50/52	1726.5
P 5	6585.8	P 43	2722.5	P 54/61	1087.9
P 6	3444.7	P 56	1121.1		
P 39	4548.4	P 57	656.1		
P 44	6291.5	P 58	1476.0		
		P 62	1113.1		
mean + std. dev.		4622.4 ± 1474.3		2065.9 ± 1482.0		1187.5 ± 496.6
median		3996.6		1476.0		1087.9

Another differentially expressed nucleic acid sequence identified codes for ILT1 receptor (immunoglobulin-like transcript 1), see SEQ ID NOs. 11 and 12. ILT1 receptor belongs to the Ig superfamily receptors that is related to a subset of activating receptors similar to NK cell receptors for MHC class I molecules. ILT1 receptor is a 69 kDa glycosylated transmembrane receptor which is mainly expressed in lung and liver and in monocytes, granulocytes, macrophages, and dendritic cells (Samaridis and Colonna 1997). Upon crosslinking with antibodies ILT1 receptor interacts with the γ-chain of the Fc receptor (FcεRIγ) (Nakajima et al. 1999)

ILT1 receptor is found consistently upregulated (59,5%) in COPD smokers compared to healthy smokers. This is demonstrated by „avg diff" values (Table 10). The p value for the comparisons between COPD smokers and healthy smokers was 0.01.

Table 10: Expression levels of ILT1 receptor: "avg diff" values for each patient are listed as well as mean and median values for the three groups of subjects; OS means obstructed smoker, HS healthy smoker, NS non-smoker

OS	avgdiff	HS	avg diff	NS	avg diff
P 1	493.5	**P 2**	412.3	**P 48/49**	519.7
P 3	1186.0	**P 37**	457.2	**P 50/52**	645.0
P 5	1097.1	**P 43**	382.6	**P 54/61**	491.2
P 6	1387.6	**P 56**	180.5		
P 39	513.5	**P 57**	367.8		
P 44	1374.5	**P 58**	720.8		
		P 62	279.1		
mean + std. dev.	1008.8 ± 406.8		400.0 ± 168.6		552.0 ± 81.8
median	1141.6		382.6		519.7

Another differentially expressed nucleic acid sequence identified codes for SHPS-1 receptor (SIRP-alpha1, MYD1, MFR), see SEQ ID NOs. 3 and 4, which is known to be highly expressed in macrophages (Fujioka et al. 1996, Kharitonenkov et al. 1997, Brooke et al. 1998). SHPS-1 receptor is a transmembrane glycoprotein belonging to immunoglobulin superfamily. It contains three extracellular Ig-like domains, a cytoplasmic tail with a potential tyrosine phosphorylation site and an immunoreceptor tyrosine-based inhibitory motif (ITIM). Tyrosine phosphorylation of SHPS-1 receptor occurs upon activation of receptor tyrosine kinases and leads to an association with SHP-1 (in macrophages) and SHP-2 (in non-hematopoetic cells) (Veillette et al. 1998). Moreover, other proteins have been found to associate with the intracytoplasmic domain of SHPS-1 receptor, and it is therefore tempting to assume that SHPS-1 receptor acts as a scaffolding protein.

SHPS-1 receptor is consistently found downregulated (73.8%) in COPD smokers compared to healthy smokers. This is demonstrated by calculated „fold change" values (Table 11) from 42 pairwise comparisons and by „avg diff" values (Table 12). The p value for the comparisons between COPD smokers and healthy smokers is 0.005.

Table 11. Expression pattern for SHPS-1 receptor: fold change calculation for 42 pairwise comparisons between COPD and healthy smokers. Only values higher than 2fold and lower than –2fold are considered as deregulated. Thus, SHPS-1 receptor is 29 times downregulated and 13 times not regulated

fold change	comparison	fold change	comparison	fold change	comparis.
-1.3	39vs2	-3.4	5vs2	1.3	1vs2
-2.8	39vs37	-6.8	5vs37	-1.7	1vs37
-1.6	39vs43	-8.4	5vs43	-2.1	1vs43
-3.0	39vs56	-7.1	5vs56	-1.8	1vs56
-5.6	39vs57	-13.2	5vs57	-3.4	1vs57
-5.4	39vs58	-12.6	5vs58	-3.2	1vs58
-3.1	39vs62	-7.5	5vs62	-1.9	1vs62
1.4	44vs2	-2.1	6vs2	-1.1	3vs2
-1.5	44vs37	-4.5	6vs37	-2.3	3vs37
-1.8	44vs43	-5.6	6vs43	-2.9	3vs43
-1.6	44vs56	-4.7	6vs56	-2.4	3vs56
-2.6	44vs57	-8.9	6vs57	-4.6	3vs57
-2.5	44vs58	-8.5	6vs58	-4.4	3vs58
-1.7	44vs62	-4.9	6vs62	-2.5	3vs62

Table 12: Expression levels of SHPS-1 receptor: "avg diff" values for each patient are listed as well as mean and median values for the three groups of subjects; OS means obstructed smoker, HS healthy smoker, NS non-smoker

OS	avg diff	HS	avg diff	NS	avg diff
P 1	1837.8	P 2	1442.6	P 48/49	4979.9
P 3	1361.1	P 37	3115.0	P 50/52	1120,5
P 5	291.1	P 43	3897.3	P 54/61	2090.6
P 6	696.3	P 56	3280.8		
P 39	1105.4	P 57	6220.7		
P 44	2466.0	P 58	5928.9		
		P 62	3431.7		
mean + std. dev.		1293.0 ± 783.9		3902.4 ± 1671.3		2730.3 ± 2007.7
median		1233.4		3431.7		2090.6

Another differentially expressed nucleic acid sequence identified codes for KDEL receptor 1, see SEQ ID NOs. 7 and 8, which is a receptor that has important functions in protein folding and assembly in the endoplasmic reticulum. It recognizes soluble proteins with the amino acid sequence K-D-E-L and retrieves these proteins after binding to the endoplasmic reticulum (Townsley et al. 1993). KDEL receptor 1 may be involved in the regulation of protein transport in the Golgi complex. Upon binding of a ligand the KDEL receptor dimerizes and interacts with ARF GAP (GTPase-activating protein for the ADP-ribosylation factor) (Aoe et al 1997).
It is consistently found downregulated (71.4%) in COPD smokers compared to healthy smokers. This is shown by „avg diff" values (Table 13). The p value for the comparisons between COPD smokers and healthy smokers is 0.003.

Table 13: Expression levels of KDEL receptor 1: "avg diff" values for each patient are listed as well as mean and median values for the three groups of subjects; OS means obstructed smoker, HS healthy smoker, NS non-smoker

OS	avg diff	HS	avg diff	NS	avg diff
P 1	877.6	P 2	930.6	P 48/49	1532.9
P 3	1227.2	P 37	2151.4	P 50/52	786.4
P 5	870.6	P 43	1628.6	P 54/61	1571.5
P 6	1188.6	P 56	2232.9		
P 39	1404.5	P 57	2295.1		
P 44	798.1	P 58	2364.1		
		P 62	2092.0		
mean + std. dev.		1061.1 ± 245.3		1956.4 ± 512.1		1296.9 ± 442.6
median		1033.1		2151.4		1532.9

Another differentially expressed nucleic acid sequence identified codes for the macrophage colony-stimulating factor-1 receptor precursor (CSF-1 receptor, c-fms); see SEQ ID NOs. 9 and 10. The CSF-1 receptor belongs to the subfamily of receptor tyrosine kinases. Activation of the CSF-1 receptor results in complex formation of multiple proteins, e.g. CSF-1 receptor, Shc, PI3K, Grb2, Cbl, SHP-1, Src. Moreover, ligand binding also triggers rapid tyrosine phosphorylation of a plethora of cytoplasmic proteins like Cbl, STAT3, STAT5a, STAT5b, p85PI3K, SHP-1, Vav and proteins involved in cytoskeletal organization (Yeung et al. 1998). CSF-1 receptor regulates survival, proliferation, differentiation and morphology of mononuclear phagocytes (Hampe et al. 1989).

CSF-1 receptor is consistently found downregulated (45.2%) in COPD smokers compared to healthy smokers. This is shown by „avg diff" values (Table 14). The p value for the comparisons between COPD smokers and healthy smokers is 0.002.

Table 14: Expression levels of CSF-1 receptor: "avg diff" values for each patient are listed as well as mean and median values for the three groups of subjects; OS means obstructed smoker, HS healthy smoker, NS non-smoker

OS	avg diff	HS	avg diff	NS	avg diff
P 1	1136.0	P 2	2591.4	P 48/49	2967.7
P 3	2262.5	P 37	3070.6	P 50/52	2041.6
P 5	829.5	P 43	2799.2	P 54/61	2376.4
P 6	1720.3	P 56	3293.1		
P 39	1860.7	P 57	3703.4		
P 44	1334.1	P 58	1904.9		
		P 62	2144.5		
mean + std. dev.		1523.9 ± 522.7		2786.7 ± 633.2		2461.9 ± 468.9
median		1527.2		2799.2		2376.4

1.6. Use of TaqMan Analysis for Validation of DNA-Chip Data and Diagnosis

mRNA-expression profiles obtained by DNA-chips are validated by TaqMan analysis with the same RNA preparations. Moreover, the method is also applied to determine mRNA-levels for FPRL-1 receptor in cultured cell lines and in cells isolated from human beings in order to monitor the progress of the disease.

Total RNA isolated from U937-cells that were treated for 3 days with 10 nM retinoic acid is used in order to optimize of reaction conditions for determining the mRNA-levels of FPRL-1 receptor and setting standard curves for FPRL-1 receptor and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a housekeeping gene. Quantification of FPRL-1 receptor is done with the following primers: Forward primer (FP) see SEQ ID NO. 17, Reverse primer (RP) see SEQ ID NO. 18 and TaqMan probe (TP) see SEQ ID NO.19 labeled with reporter dye FAM at the 5' end and quencher dye TAMRA at the 3' end. For determining mRNA-levels for GAPDH a predeveloped kit "TaqMan GAPDH Control Reagents" (P/N 402869) from Perkin Elmer Applied Biosystems is used. The GAPDH probe is labeled with JOE as the reporter dye and TAMRA as the quencher dye. RT-PCR reactions are performed with the "TaqMan EZ RT-PCR Core Reagents" (P/N N808-0236) kit from Perkin Elmer Applied Biosystems. Standard curves for FPRL-1 receptor and GAPDH are performed with increasing concentrations of RNA from U937 cells treated with 1 μM

retinoic acid ranging from 0, 5, 10, 25, 50 to 100 ng per assay. Reaction mixes contain 1x TaqMan EZ-buffer, 3 mM $Mn(Oac)_2$, 300 µM dATP, dCTP, dGTP, and 600 µM dUTP, 2.5 U rTth DNA polymerase, 0.25 U AmpErase UNG in a total volume of 25 µl. For analysis of FPRL-1 receptor reaction mixes include 300 nM of FP and RP and 100 nM of TP. The primer concentrations for determining GAPDH levels are 200 nM for each primer and 100 nM for the GAPDH Taqman probe. In order to determine mRNA levels for FPRL-1 receptor and GAPDH in human subjects and cell lines 16 to 50 ng RNA per reaction are used. All samples are run in triplicate. The reactions are performed with "MicroAmp Optical 96-well reaction plates" sealed with "MicroAmp Optical Caps" (Perkin Elmer Applied Biosystems) in an ABI PRISM 7700 Sequence Detection System (Perkin Elmer Applied Biosystems). The PCR conditions are 2 minutes at 50°C, 30 minutes at 60°C, 5 minutes at 95°C, followed by 40 cycles of 20 seconds at 94°C and 1 minute at 59°C. Data analysis is done either by determining the mRNA levels for FPRL-1 receptor and GAPDH according to the standard curves or by directly relating C_T values for FPRL-1 receptor to C_T values for GAPDH. The latter can be done for these genes since the efficiencies for both reactions are around 95%. The same method is used for investigating mRNA levels isolated from COPD patients in order to diagnose the disease or, after treatment of patients with their putative active drugs to monitor the success of the treatment.

The other receptors mentioned in example 1.5 are investigated accordingly by using the respective appropriate primers.

1.7. Cell Systems

Human monocytic/macrophage cell lines HL-60, U937, THP-1, and MonoMac 6 are used as cellular model systems. Cells are grown in RPMI 1640 media containing 10% FCS supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 1x non-essential amino acids. The media for MonoMac6 cells also includes 5 ml/l OPI media supplement (Sigma). MonoMac6 cells are exclusively cultured in 24-well plates. Cells are maintained in a humidified atmosphere with 5% CO_2 at 37°C and tested regularly for contamination by mycoplasma. Differentiation is achieved by adding 10 nM PMA (phorbol 12 myristate-13 acetate) to the media.

1.8. Cloning of FPRL-1 receptor

FPRL-1 receptor is cloned from a total RNA extracted from U937 cells that were treated with 1 µM retinoic acid for three days. 5 µg RNA is reverse transcribed into cDNA with 5 ng oligo$(dt)_{18}$ primer, 1x first strand buffer, 10 mM DTT, 0.5 mM dNTPs and 2 U Superscript II (Gibco BRL Life Technologies) at 42°C for 50 minutes. Then, the reaction is terminated at 70°C for 15 minutes and the cDNA concentration is

determined by UV-spectrophotometry. For amplification of FPRL-1 receptor 100 ng of the cDNA and 10 pmol of sequence-specific primers for FPRL-1 receptor (forward primer attB1; see SEQ ID NO. 15 and reverse primer attB2 ; see SEQ ID NO. 16) are used for PCR. Reaction conditions are: 2 minutes of 94°C, 35 cycles with 30 seconds at 94°C, 30 seconds at 53°C, 90 seconds at 72°C, followed by 7 minutes at 72°C with Taq DNA-polymerase. The reaction mix is separated on a 2% agarose gel, a band of about 1000bp is cut out and purified with the QIAEX II extraction kit (Qiagen). The concentration of the purified band is determined and about 120 ng are incubated with 300 ng of pDONR201, the donor vector of the Gateway system (Gibco BRL Life Technologies), 1x BP clonase reaction buffer, BP clonase enzyme mix in a total volume of 20 µl for 60 minutes at 25°C. Then, reactions are incubated with 2 µl of proteinase K and incubated for 10 minutes at 37°C. The reaction mix is then electroporated into competent DB3.1 cells and plated on Kanamycin-containing plates. Clones are verified by sequencing. A clone, designated pDONR-HM63 carrying the nucleic acid sequence shown in SEQ ID No. 1 is used for further experiments.

The other receptors mentioned in example 1.5 are cloned using analogous methods.

1.9. Transfection of FPRL-1 receptor

The vector containing FPRL-1 receptor described under 1.8 is used to transfer the cDNA for FPRL-1 receptor to the expression vector pcDNA3.1(+)/attR that contains the "attR1" and "attR2" recombination sites of the Gateway cloning system (Gibco BRL Life Technologies) where FPRL-1 receptor is expressed under the control of the CMV promoter. 150 ng of the "entry vector" pDONR-HM63 is mixed with 150 ng of the "destination vector" pcDNA3.1(+)/attR, 4 µl of the LR Clonase enzyme mix, 4 µl LR Clonase reaction buffer, added up with TE (Tris/EDTA) to 20 µl and incubated at 25°C for 60 minutes. Then, 2 µl of proteinase K solution is added and incubated for 10 minutes at 37°C. 1 µl of the reaction mix is transformed into 50 µl DH5α by a heat-shock of 30 seconds at 42°C after incubating cells with DNA for 30 minutes on ice. After heat-shock of the cells 450 µl of S.O.C. is added and cells are incubated at 37°C for 60 minutes. Cells (100 µl) are plated on LB plates containing 100 µg/ml ampicillin and incubated over night.

A colony that contains pcDNA3.1(+)/attR with FPRL-1 receptor as an insert is designated pcDNA/FPRL1 and used for transfection studies.

Cell clones containing vectors obtained in 1.8 carrying nucleic acid sequences coding for the other receptors described 1.5 are prepared using analogous methods.

Example 2: Cellular Systems and Phenotypic Effects of FPRL-1 receptor

Analogous methods as described herein in example 2 for FPRL-1 receptor are also performed using the other receptors described in 1.5.

2.1. Cell Systems

Human monocytic/macrophage cell lines HL-60, U937, THP-1, and MonoMac6 are used as cellular model systems. Cells are grown in RPMI 1640 media containing 10% FCS supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 1x non-essential amino acids. The media for MonoMac6 also includes 5 ml/l OPI media supplement (Sigma). MonoMac6 cells are exclusively cultured in 24-well plates. All cells are maintained in a humidified atmosphere with 5% CO_2 at 37°C and tested regularly for contamination by mycoplasma. Differentiation is achieved by adding 10 nM PMA (phorbol 12 myristate-13 acetate) to the media.

Phenotypic effects of FPRL-1 receptor (2.2.-2.9.)

2.2. Ligand Binding Assay

300 ml cell culture is harvested with EDTA solution, the suspension is used to spin down the cells at 110-220 x g, resuspended in 10 mM Tris/HCl, pH 7.4, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 40 µg/ml bacitracin, 4 µg/ml leupeptin, 4 µg/ml chymostatin, 10 µg/ml pefabloc, 2 µM phosphoamidon and 0,1 mg/ml bovine serum albumin (BSA Fraktion V, BI Bioproducts) and diluted to 2×10^6 cells/ml.

0.5 ml aliquots are incubated with 0.3 nM ^3H-lipoxinA4 (specific activity ~10 Ci/mmol) or in the presence of increasing concentrations of untritiated lipoxin A4 (3-300 nM) for 30 minutes at 4°C. The incubation is terminated by harvesting the cells by a Cell-Harvester (Skatron) with GF/B filters, washed three times with 3 ml chilled buffer consisting of 50 mM Tris/HCl, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4 and the filter-pieces transferred in vials. 2 ml scintillation cocktail is added and the radioactivity determined with a scintillation counter (LKB). Non-specific binding is determined in the presence of 100 nM unlabeled lipoxinA4. A series of peptides and low molecular weight compounds, including the peptide ligand MMK-1 (Klein et al. 1998), is used in a concentration range of 0.5 to 300 nM under the same reaction conditions in order to displace tritiated lipoxin A4.

The bound radioactivity (on the filter pieces) is estimated with a counter, the values are recorded on-line and fitted to a model. IC_{50} values for any substance to block binding of ^3H-lipoxin A4 are calculated.

2.3. Ca^{2+}-Release Determined by FLIPR-Assay

FLIPR-assay (Fluorometric Imaging Plate Reader) with FPRL-1 receptor is performed with a CHO cell line that constitutively expresses the G-protein α-subunit α16 and FPRL-1 receptor. The cell line CHO/Galpha16 (CHO/Galpha16) (Boehringer Ingelheim) that constitutively expresses Gα16 is transfected with the FPRL-1 receptor expression vector. CHO/Galpha16 are cultured in Ham's F12 media (Bio Whittaker) with 10% FCS (fetal calf serum), 2 mM glutamine, 200 ng/ml hygromycin, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37°C. 3-7x10^5 cells of CHO/Galpha16 are seeded in a 60 mm petri dish and grown over night. Cells that are grown to a confluency of 50-80% are used for transfection. 6 µl FuGene6 (Roche Biochemicals) is added to 100 µl of culture media without serum and equilibrated for 5 minutes at room temperature. Then, 2 µg of purified pcDNA/FPRL-1 receptor is added to the prediluted FuGene6 solution, gently mixed, and further incubated at room temperature for 15 minutes. The media is aspirated from the cells and 4 ml of fresh media is added to the cells. The FuGene6/DNA solution is added dropwise to the cells and distributed evenly by swirling of the media. After 48 hours the media is aspirated and replaced by Ham's F12 media, 10% FCS, 2 mM glutamine, 200 ng/ml hygromycin, 100 U/ml penicillin,100 µg/ml streptomycin, and 200 µg/ml G418. During the following five days the media is replaced daily until dead cells and debris is washed out until single colonies of cells are visible. Single colonies are isolated by separation with cloning cylinders and releasing them from the surface by addition of 100 µl of 1x trypsin/EDTA. Cells are transferred from the cloning cylinders to 4 ml of media and plated in 6 well-plates. Single clones are expanded and the expression of FPRL-1 receptor in several clones is tested via ligand binding assay (2.2.). The cell clone denoted CHO/Galpha16/FPRL-1 receptor with the highest expression of FPRL-1 receptor is used for measuring of intracytoplasmic Ca^{2+} via FLIPR (Molecular Devices).

Cells (CHO/Galpha16/FPRL-1 receptor) are seeded in 384-blackwell plates (Corning) with 2500-5000 cells per well in a volume of 40 µl and grown over night in a humidified atmosphere with 5% CO_2 at 37°C. As a negative control CHO/Galpha16 cells are used. Then, 40 µl of a Fluo-4 (Molecular Probes) staining solution is added to each well in order to label the cells with Fluo-4 at a final concentration of 2 µM. The Fluo-4 staining solution is composed of 10.5 ml cell culture media described above, 420 µl Probenicid solution (1.42 g Probenicid (Sigma), 10 ml 1 M NaOH, 10 ml Hanks buffer), 42 µl Fluo-4 stock solution (50 µg Fluo-4, 23 µl DMSO, 23 µl Pluronic F-127 (20% in DMSO) (Molecular Probes), and 420 µl 1M HEPES. After 45 minutes incubation in a humidified atmosphere with 5% CO_2 at 37°C wells are washed with a EMBLA-washer (4 wash steps, program 03) using 2000 ml Hanks

buffer containing 20 ml Probenicid solution as a wash solution and leaving 25 µl wash buffer in each well. Then FLIPR is set to 10000 counts for stained wells and a difference of 1:5 between unstained and stained wells. Then, 25 µl lipoxin A4 and a series of ligands, peptids, and low molecular weight compounds, including the peptide ligand MMK-1 is added to the wells in increasing concentrations (0.5 - 300 nM) diluted in Hanks' buffer/0.1% BSA. Substances according to the invention are tested in increasing concentrations (0.5 - 300 nM) to compete with lipoxin A4 (50 nM) in order to determine their antagonistic potential. Fluorescence is recorded starting with the addition of the ligand every second for 60 seconds and every 5 seconds for a further 60 seconds.

2.4. Production and Release of Cytokines or Matrix Metalloproteases

Cells of monocytic/macrophage cell lines are treated with lipoxin A4 at cell densities between 2.5 and 5 x 10^5 cells/ml. Cells are harvested after 0, 1, 3, 6, 12, 24, 48, and 72 hours, the supernatant frozen for further investigation, cells are washed with PBS, and resuspended in 400 ml of RLT buffer (from Qiagen RNeasy Total RNA Isolation Kit) with 143 mM β-mercaptoethanol, the DNA sheared with a 20 g needle for at least 5 times and stored at –70°C.

Total RNAs are isolated with the Qiagen RNeasy Total RNA Isolation Kit (Qiagen) according to the manufacturer's protocol. Purified RNA is used for TaqMan analysis. The expression levels of cytokines TNFα, IL-1β, IL-8, IL-6, and human matrix metalloproteases, MMP-1, MMP-7, MMP-9, MMP-12, are measured using appropriate primer sequences.

2.4.1. Detection of Secreted Cytokines

Proteins in the supernatants of the cultured and stimulated cells are precipitated by adding TCA (tricholoracetic acid) to a final concentration of 10%. Precipitates are washed twice with 80% ethanol and pellets are resuspended in 50 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA. Protein concentration is determined via the Bradford method and 50 µg of each sample are loaded on 12% SDS polyacrylamide gels. Gels are blotted onto PVDF-membranes, blocked for 1 hour in 5% BSA in TBST, and incubated for 1 hour with commercially available antibodies against human TNFα (tumor necrosis factor α) IL-1β (interleukin-1β), IL-8 (interleukin 8), and IL-6 (interleukin 6). After washing with TBST blots are incubated with anti-human IgG conjugated to horseradish-peroxidase, washed again and developed with ECL chemiluminescence kit (Amersham). Intensity of the bands are visualised with BioMax X-ray films (Kodak) and quantified by densitometry.

2.4.2. Detection and Activity of Secreted Matrix Metalloproteases

The procedure is identical to the one described in 2.4.1. Antibodies used for Western blotting are against human MMP-1, MMP-7, MMP-9, and MMP-12.

Protease activity is determined with a fluorescent substrate. Supernatants isolated from stimulated and unstimulated cells (described above) are incubated in a total volume of 50 µl with 1 µM of the substrate (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Glu (EDANS)-Ala-Lys-NH2 (Novabiochem)) for 5 minutes at room temperature. Positive controls are performed with 125 ng purified MMP-12 per reaction. Protease activity is determined by fluorometry with an excitation at 320 nm and an emission at 405 nm.

In an alternative assay to determine proteolytic activity and cell migration a chemotaxis chamber is used. In the wells of the upper part of the chamber cells (10^5 cells per well) are plated on filters coated with an 8 µm layer of Matrigel (Becton Dickinson). In the lower compartment chemoattractants like lipoxin A4 (100 nM), MCP-1 (monocyte chemotactic protein 1) (10 ng/ml) are added to the media. After five days filters are removed, cells on the undersurface that have traversed the Matrigel are fixed with methanol, stained with the Diff-Quik staining kit (Dade Behring) and counted in three high power fields (400x) by light microscopy.

2.5. Chemotaxis Assay

In order to determine chemotaxis a 48 well chemotaxis (Boyden) chamber (Neuroprobe) is used. Cells are starved for 24 hours in RPMI media without FCS. Chemoattractants, (50 ng/ml IL-8 , 10 ng/ml MCP-1, 10 nM lipoxin A4, 10 nM MMK-1 peptide (2.3.)) are diluted in RPMI media without FCS and 30 µl is placed in the wells of the lower compartment. The upper compartment is separated from the lower compartment by a polycarbonate filter (pore size 8 µm). 50 µl cell suspension (5 $x10^4$) are placed in the well of the upper compartment. The chamber is incubated for 5 hours at 37°C in a humidified atmosphere with 5% CO_2. Then the filter is removed, cells on the upper side are scraped off, cells on the downside are fixed for 5 minutes in methanol and stained with the Diff-Quik staining set (Dade Behring). Migrated cells are counted in three high-power fields (400x) by light microscopy.

2.6. Adherence Assay

Cells are harvested, washed in PBS and resuspended ($4x10^6$/ml) in PBS and 1 µM BCECF ((2'-7'-bis-(carboxethyl)-5(6')-carboxyfluorescein acetoxymethyl) ester, Calbiochem) and incubated for 20 minutes at 37°C. Cells are washed in PBS and resuspended ($3.3x\ 10^6$/ml) in PBS containing 0.1% BSA. $3x10^5$ cells (90 µl) are added to each well of a 96-well flat bottom plate coated with laminin (Becton

Dickinson) and allowed to settle for 10 minutes. 10 µl of agonist (100 nM lipoxin A4 plus lipoxin A4 antagonist) are added and plates are incubated for 20 minutes at 37°C. Then, cells are washed with PBS containing 0.1% BSA and adherent cells are solubilized with 100 µl of 0.025 M NaOH and 0.1% SDS. Quantification is performed by fluorescence measurement.

2.7. Phagocytosis

Cell suspensions ($2.5x10^4$ cells/ml) are seeded in 6-well plates with 5 ml of U937 or THP-1 or in 24-well plates with 2 ml of MonoMac6 and incubated for 1 hour at 37°C in a humidified atmosphere with 5% CO_2 in the presence of agonists (100 nM lipoxin A4, 50 nM MMK-1 peptide (2.3.)) and low molecular weight compounds according to the invention in order to antagonize agonistic effects. 40 µl of a dispersed suspension of heat-inactivated Saccharomyces boulardii (20 yeast/cell) are added to each well. Cells are incubated for three more hours, washed twice with PBS and cytocentrifuged. The cytospin preparations are stained with May-Grünwald-Giemsa and phagocytosed particles are counted by light microsopy.

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Claims

1) A method for determining whether a substance is an activator or an inhibitor of an ILM receptor

2) A method according to claim 1 in which said ILM receptor is a mammalian receptor.

3) A method according to claim 2 in which said ILM receptor is a human receptor.

4) A method according to claim 1 in which the analysis is performed using a cellular system.

5) A method according to claim 1 in which the analysis is performed using a cell-free system.

6) A method according to claim 1 in which said receptor is a receptor selected from the group consisting of FPRL-1 receptor type receptor including FPRL-1 receptor (SEQ ID NO. 2); HM74 receptor type receptor including HM74 receptor (SEQ ID NO. 21); AICL receptor type receptor including AICL receptor (SEQ ID NO. 6)); ILT1 receptor type receptor including ILT1 receptor (SEQ ID NO. 12); SHPS receptor type receptor including SHPS-1 receptor (SEQ ID NO. 4); KDEL receptor 1 type receptor including KDEL receptor 1 (SEQ ID NO. 8); and CSF-1 receptor type receptor including CSF-1 receptor (SEQ ID NO. 10).

7) A method according to claim 6 in which said receptor is a FPRL-1 receptor type receptor.

8) A method according to claim 7 in which the FPRL-1 receptor type receptor of SEQ ID NO 2 is used or a variant, mutant, or fragment thereof having the same function.

9) A method for determining an expression level of an ILM receptor comprising determining the level of ILM receptor expressed in a macrophage.

10) A method according to claim 9 in which said macrophage is a mammalian cell.

11) A method according to claim 10 in which said macrophage is a human cell.

12) A method according to claim 11 in which the analysis is performed using a macrophage or a part thereof obtainable from the site of inflammation.

13) A method according to claim 12 in which the analysis is performed using a macrophage or a part thereof obtainable from the site of inflammation in a mammal.

14) A method according to claim 13 in which the analysis is performed using a macrophage or a part thereof obtainable from the site of inflammation in a human being.

15) A method according to claim 9 in which said receptor is a receptor selected from the group consisting of FPRL-1 receptor type receptor including FPRL-1 receptor (SEQ ID NO. 2); HM74 receptor type receptor including HM74 receptor (SEQ ID NO. 21); AICL receptor type receptor including AICL receptor (SEQ ID NO. 6)); ILT1 receptor type receptor including ILT1 receptor (SEQ ID NO. 12); SHPS receptor type receptor including SHPS-1 receptor (SEQ ID NO. 4); KDEL receptor 1 type receptor including KDEL receptor 1 (SEQ ID NO. 8); and CSF-1 receptor type receptor including CSF-1 receptor (SEQ ID NO. 10).

16) A method according to claim 9 in which said receptor is a FPRL-1 receptor type receptor.

17) A method according to claim 16 in which the FPRL-1 receptor type receptor of SEQ ID NO 2 is used or a variant, mutant, or fragment thereof having the same function.

18) A method according to claim 9 for diagnosis or monitoring of a chronic inflammatory airway disease.

19) A method according to claim 18 in which the chronic inflammatory airway disease is selected from the group consisting of chronic bronchitis and COPD.

20) A method according to claim 18 in which the analysis is performed using a macrophage or a part thereof obtainable from the site of inflammation.

21) A method according to claim 20 in which the analysis is performed using a macrophage or a part thereof obtainable from the site of inflammation in a mammal.

22) A method according to claim 21 in which the analysis is performed using a macrophage or a part thereof obtainable from the site of inflammation in a human being.

23) A test system for determining whether a substance is an activator or an inhibitor of an ILM receptor function characterized in that the receptor is involved in a chronic inflammatory airway disease and which receptor plays a role in mediating inflammation.

24) A test system according to claim 23 in which said receptor is a receptor selected from the group consisting of FPRL-1 receptor type receptor including FPRL-1 receptor (SEQ ID NO. 2); HM74 receptor type receptor including HM74 receptor (SEQ ID NO. 21); AICL receptor type receptor including AICL receptor (SEQ ID NO. 6)); ILT1 receptor type receptor including ILT1 receptor (SEQ ID NO. 12); SHPS receptor type receptor including SHPS-1 receptor (SEQ ID NO. 4); KDEL receptor 1 type receptor including KDEL receptor 1 (SEQ ID NO. 8); and CSF-1 receptor type receptor including CSF-1 receptor (SEQ ID NO. 10).

25) A test system according to claim 23 in which said receptor is a FPRL-1 receptor type receptor.

26) A test system according to claim 25 in which the FPRL-1 receptor type receptor of SEQ ID NO 2 is used or a variant, mutant, or fragment thereof having the same function.

27) A test system according to claim 26 comprising a cell expressing an ILM receptor.

28) A substance determined to be an activator or inhibitor of an ILM receptor.

29) A substance according to claim 28 in which said receptor is a receptor selected from the group consisting of FPRL-1 receptor type receptor including FPRL-1 receptor (SEQ ID NO. 2); HM74 receptor type receptor including HM74

receptor (SEQ ID NO. 21); AICL receptor type receptor including AICL receptor (SEQ ID NO. 6)); ILT1 receptor type receptor including ILT1 receptor (SEQ ID NO. 12); SHPS receptor type receptor including SHPS-1 receptor (SEQ ID NO. 4); KDEL receptor 1 type receptor including KDEL receptor 1 (SEQ ID NO. 8); and CSF-1 receptor type receptor including CSF-1 receptor (SEQ ID NO. 10).

30) A substance according to claim 28 in which said receptor is a FPRL-1 receptor type receptor.

31) A substance according to claim 30 in which the FPRL-1 receptor type receptor of SEQ ID NO 2 is used or a variant, mutant, or fragment thereof having the same function.

32) A substance which is an activator or inhibitor of an ILM receptor for the treatment of a disease.

33) A substance according to claim 32 in which said receptor is a receptor selected from the group consisting of FPRL-1 receptor type receptor including FPRL-1 receptor (SEQ ID NO. 2); HM74 receptor type receptor including HM74 receptor (SEQ ID NO. 21); AICL receptor type receptor including AICL receptor (SEQ ID NO. 6)); ILT1 receptor type receptor including ILT1 receptor (SEQ ID NO. 12); SHPS receptor type receptor including SHPS-1 receptor (SEQ ID NO. 4); KDEL receptor 1 type receptor including KDEL receptor 1 (SEQ ID NO. 8); and CSF-1 receptor type receptor including CSF-1 receptor (SEQ ID NO. 10).

34) A substance according to claim 32 in which said receptor is a FPRL-1 receptor type receptor.

35) A substance according to claim 34 in which the FPRL-1 receptor type receptor of SEQ ID NO 2 is used or a variant, mutant, or fragment thereof having the same function.

36) A substance according to claim 32 in which said disease is a chronic inflammatory airway disease.

37) A substance according to claim 36 in which said chronic inflammatory airway disease is selected from the group consisting of chronic bronchitis and COPD.

38) A pharmaceutical composition comprising at least one substance determined to be an activator or inhibitor of an ILM receptor

39) A pharmaceutical composition according to claim 38 in which said receptor is a receptor selected from the group consisting of FPRL-1 receptor type receptor including FPRL-1 receptor (SEQ ID NO. 2); HM74 receptor type receptor including HM74 receptor (SEQ ID NO. 21); AICL receptor type receptor including AICL receptor (SEQ ID NO. 6)); ILT1 receptor type receptor including ILT1 receptor (SEQ ID NO. 12); SHPS receptor type receptor including SHPS-1 receptor (SEQ ID NO. 4); KDEL receptor 1 type receptor including KDEL receptor 1 (SEQ ID NO. 8); and CSF-1 receptor type receptor including CSF-1 receptor (SEQ ID NO. 10).

40) A pharmaceutical composition according to claim 38 in which said receptor is a FPRL-1 receptor type receptor.

41) A pharmaceutical composition according to claim 40 in which the FPRL-1 receptor type receptor of SEQ ID NO 2 is used or a variant, mutant, or fragment thereof having the same function.

42) Use of a substance determined to be an activator or inhibitor of an ILM receptor for preparing a pharmaceutical composition for treating a chronic inflammatory airway disease.

43)Use of a substance according to claim 42 in which the chronic inflammatory airway disease is selected from the group consisting of chronic bronchitis and COPD.

44) A use according to claim 42 in which said receptor is a receptor selected from the group consisting of FPRL-1 receptor type receptor including FPRL-1 receptor (SEQ ID NO. 2); HM74 receptor type receptor including HM74 receptor (SEQ ID NO. 21); AICL receptor type receptor including AICL receptor (SEQ ID NO. 6)); ILT1 receptor type receptor including ILT1 receptor (SEQ ID NO. 12); SHPS receptor type receptor including SHPS-1 receptor (SEQ ID NO. 4); KDEL receptor 1 type receptor including KDEL receptor 1 (SEQ ID NO. 8); and CSF-1 receptor type receptor including CSF-1 receptor (SEQ ID NO. 10).

45) A use according to claim 42 in which said receptor is a FPRL-1 receptor type receptor.

46) A use according to claim 45 in which the FPRL-1 receptor type receptor of SEQ ID NO 2 is used or a variant, mutant, or fragment thereof having the same function.

47)A method for treating a chronic inflammatory airway disease which method comprises administering to a being in need of such treatment a suitable amount of a pharmaceutical composition comprising at least one substance determined to be an activator or inhibitor of an ILM receptor

48) A method according to claim 47 in which said receptor is a receptor selected from the group consisting of FPRL-1 receptor type receptor including FPRL-1 receptor (SEQ ID NO. 2); HM74 receptor type receptor including HM74 receptor (SEQ ID NO. 21); AICL receptor type receptor including AICL receptor (SEQ ID NO. 6)); ILT1 receptor type receptor including ILT1 receptor (SEQ ID NO. 12); SHPS-1 receptor type receptor including SHPS receptor (SEQ ID NO. 4); KDEL receptor 1 type receptor including KDEL receptor 1 (SEQ ID NO. 8); and CSF-1 receptor type receptor including CSF-1 receptor (SEQ ID NO. 10).

49) A method according to claim 47 in which said receptor is a FPRL-1 receptor type receptor.

50) A method according to claim 49 in which the FPRL-1 receptor type receptor of SEQ ID NO 2 is used or a variant, mutant, or fragment thereof having the same function.

51)A method according to claim 47 for treating a mammal

52) A method according to claim 47 for treating a human being

53) A method according to claim 47 for treating a chronic inflammatory airway disease selected from the group consisting of chronic bronchitis and COPD.

54) A method for selectively modulating an ILM receptor in a macrophage, comprising administering a substance determined to be an activator or inhibitor of an ILM receptor.

55) A method according to claim 54 in which the macrophage is involved in a chronic inflammatory airway disease

56) A method according to claim 55 in which the chronic inflammatory airway disease is selected from the group consisting of chronic bronchitis and COPD

57) A method according to claim 54 in which said receptor is a receptor selected from the group consisting of FPRL-1 receptor type receptor including FPRL-1 receptor (SEQ ID NO. 2); HM74 receptor type receptor including HM74 receptor (SEQ ID NO. 21); AICL receptor type receptor including AICL receptor (SEQ ID NO. 6)); ILT1 receptor type receptor including ILT1 receptor (SEQ ID NO. 12); SHPS-1 receptor type receptor including SHPS-1 receptor (SEQ ID NO. 4); KDEL receptor 1 type receptor including KDEL receptor 1 (SEQ ID NO. 8); and CSF-1 receptor type receptor including CSF-1 receptor (SEQ ID NO. 10).

58) A method according to claim 54 in which said receptor is a FPRL-1 receptor type receptor.

59) A method according to claim 58 in which the FPRL-1 receptor type receptor of SEQ ID NO 2 is used or a variant, mutant, or fragment thereof having the same function.

Abstract

The present invention relates to substances which modulate receptors involved in inflammatory processes and whose modulated functions positively influence inflammatory diseases.

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<211> 503
<212> PRT
<213> Homo sapiens

<400> 4

Met Glu Pro Ala Gly Pro Ala Pro Gly Arg Leu Gly Pro Leu Leu Cys
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Glu Leu Gln Val Ile Gln Pro Asp Lys Ser Val Ser Val Ala Ala Gly
35 40 45

Glu Ser Ala Ile Leu His Cys Thr Val Thr Ser Leu Ile Pro Val Gly
50 55 60

Pro Ile Gln Trp Phe Arg Gly Ala Gly Pro Ala Arg Glu Leu Ile Tyr
65 70 75 80

Asn Gln Lys Glu Gly His Phe Pro Arg Val Thr Thr Val Ser Glu Ser
85 90 95

Thr Lys Arg Glu Asn Met Asp Phe Ser Ile Ser Ile Ser Asn Ile Thr
100 105 110

Pro Ala Asp Ala Gly Thr Tyr Tyr Cys Val Lys Phe Arg Lys Gly Ser
115 120 125

Pro Asp Thr Glu Phe Lys Ser Gly Ala Gly Thr Glu Leu Ser Val Arg

130 135 140

Ala Lys Pro Ser Ala Pro Val Val Ser Gly Pro Ala Ala Arg Ala Thr
145 150 155 160

Pro Gln His Thr Val Ser Phe Thr Cys Glu Ser His Gly Phe Ser Pro
165 170 175

Arg Asp Ile Thr Leu Lys Trp Phe Lys Asn Gly Asn Glu Leu Ser Asp
180 185 190

Phe Gln Thr Asn Val Asp Pro Val Gly Glu Ser Val Ser Tyr Ser Ile
195 200 205

His Ser Thr Ala Lys Val Val Leu Thr Arg Glu Asp Val His Ser Gln
210 215 220

Val Ile Cys Glu Val Ala His Val Thr Leu Gln Gly Asp Pro Leu Arg
225 230 235 240

Gly Thr Ala Asn Leu Ser Glu Thr Ile Arg Val Pro Pro Thr Leu Glu
245 250 255

Val Thr Gln Gln Pro Val Arg Ala Glu Asn Gln Val Asn Val Thr Cys
260 265 270

Gln Val Arg Lys Phe Tyr Pro Gln Arg Leu Gln Leu Thr Trp Leu Glu
275 280 285

Asn Gly Asn Val Ser Arg Thr Glu Thr Ala Ser Thr Val Thr Glu Asn
290 295 300

Lys Asp Gly Thr Tyr Asn Trp Met Ser Trp Leu Leu Val Asn Val Ser
305 310 315 320

Ala His Arg Asp Asp Val Lys Leu Thr Cys Gln Val Glu His Asp Gly
325 330 335

Gln Pro Ala Val Ser Lys Ser His Asp Leu Lys Val Ser Ala His Pro
340 345 350

Lys Glu Gln Gly Ser Asn Thr Ala Ala Glu Asn Thr Gly Ser Asn Glu

355 360 365

Arg Asn Ile Tyr Ile Val Val Gly Val Val Cys Thr Leu Leu Val Ala
370 375 380

Leu Leu Met Ala Ala Leu Tyr Leu Val Arg Ile Arg Gln Lys Lys Ala
385 390 395 400

Gln Gly Ser Thr Ser Ser Thr Arg Leu His Glu Pro Glu Lys Asn Ala
405 410 415

Arg Glu Ile Thr Gln Asp Thr Asn Asp Ile Thr Tyr Ala Asp Leu Asn
420 425 430

Leu Pro Lys Gly Lys Lys Pro Ala Pro Gln Ala Ala Glu Pro Asn Asn
435 440 445

His Thr Glu Tyr Ala Ser Ile Gln Thr Ser Pro Gln Pro Ala Ser Glu
450 455 460

Asp Thr Leu Thr Tyr Ala Asp Leu Asp Met Val His Leu Asn Arg Thr
465 470 475 480

Pro Lys Gln Pro Ala Pro Lys Pro Glu Pro Ser Phe Ser Glu Tyr Ala
485 490 495

Ser Val Gln Val Pro Arg Lys
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<210> 5
<211> 759
<212> DNA
<213> Homo sapiens

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aagaagcacg gtatgatgac caaacataaa aagtgtttta taattgttgg tgttttaata 180
acaactaata ttattactct gatagttaaa ctaactcgag attctcagag tttatgcccc 240
tatgattgga ttggtttcca aaacaaatgc tattatttct ctaaagaaga aggagattgg 300
aattcaagta aatacaactg ttccactcaa catgccgacc taactataat tgacaacata 360

gaagaaatga attttcttag gcggtataaa tgcagttctg atcactggat tggactgaag 420
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ggggaaaata gaaaataaca ttattaagtg taaaaccagc aaagtacttt tttaattaaa 660
caaagttcga gttttgtacc tgtctggtta attctgctta cgtgtcaggc tacacataaa 720
agccacttca aagattggca aaaaaaaaaa aaaaaaaaa 759

<210> 6
<211> 149
<212> PRT
<213> Homo sapiens

<400> 6

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1 5 10 15

Thr Thr Asn Ile Ile Thr Leu Ile Val Lys Leu Thr Arg Asp Ser Gln
20 25 30

Ser Leu Cys Pro Tyr Asp Trp Ile Gly Phe Gln Asn Lys Cys Tyr Tyr
35 40 45

Phe Ser Lys Glu Glu Gly Asp Trp Asn Ser Ser Lys Tyr Asn Cys Ser
50 55 60

Thr Gln His Ala Asp Leu Thr Ile Ile Asp Asn Ile Glu Glu Met Asn
65 70 75 80

Phe Leu Arg Arg Tyr Lys Cys Ser Ser Asp His Trp Ile Gly Leu Lys
85 90 95

Met Ala Lys Asn Arg Thr Gly Gln Trp Val His Gly Ala Thr Phe Thr
100 105 110

Lys Ser Phe Gly Met Arg Gly Ser Glu Gly Cys Ala Tyr Leu Ser Asp
115 120 125

Asp Gly Ala Ala Thr Ala Arg Cys Tyr Thr Glu Arg Lys Trp Ile Cys
130 135 140

Arg Lys Arg Ile His
145

<210> 7
<211> 1086
<212> DNA
<213> Homo sapiens

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<210> 8
<211> 212
<212> PRT
<213> Homo sapiens

<400> 8
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Ile Leu Leu Leu Leu Lys Ile Trp Lys Ser Arg Ser Cys Ala Gly Ile
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Ser Gly Lys Ser Gln Val Leu Phe Ala Val Val Phe Thr Ala Arg Tyr
35 40 45

Leu Asp Leu Phe Thr Asn Tyr Ile Ser Leu Tyr Asn Thr Cys Met Lys
50 55 60

Val Val Tyr Ile Ala Cys Ser Phe Thr Thr Val Trp Leu Ile Tyr Ser
65 70 75 80

Lys Phe Lys Ala Thr Tyr Asp Gly Asn His Asp Thr Phe Arg Val Glu
85 90 95

Phe Leu Val Ile Pro Thr Ala Ile Leu Ala Phe Leu Val Asn His Asp
100 105 110

Phe Thr Pro Leu Glu Ile Leu Trp Thr Phe Ser Ile Tyr Leu Glu Ser
115 120 125

Val Ala Ile Leu Pro Gln Leu Phe Met Val Ser Lys Thr Gly Glu Ala
130 135 140

Glu Thr Ile Thr Ser His Tyr Leu Phe Ala Leu Gly Val Tyr Arg Thr
145 150 155 160

Leu Tyr Leu Phe Asn Trp Ile Trp Arg Tyr His Phe Glu Gly Phe Phe
165 170 175

Asp Leu Ile Ala Ile Val Ala Gly Leu Val Gln Thr Val Leu Tyr Cys
180 185 190

Asp Phe Phe Tyr Leu Tyr Ile Thr Lys Val Leu Lys Gly Lys Lys Leu
195 200 205

Ser Leu Pro Ala
210

<210> 9
<211> 3992
<212> DNA
<213> Homo sapiens

<400> 9

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<211> 972
<212> PRT
<213> Homo sapiens

<400> 10

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Gly Gln Gly Ile Pro Val Ile Glu Pro Ser Val Pro Glu Leu Val Val
20 25 30

Lys Pro Gly Ala Thr Val Thr Leu Arg Cys Val Gly Asn Gly Ser Val

35 40 45

Glu Trp Asp Gly Pro Pro Ser Pro His Trp Thr Leu Tyr Ser Asp Gly
50 55 60

Ser Ser Ser Ile Leu Ser Thr Asn Asn Ala Thr Phe Gln Asn Thr Gly
65 70 75 80

Thr Tyr Arg Cys Thr Glu Pro Gly Asp Pro Leu Gly Gly Ser Ala Ala
85 90 95

Ile His Leu Tyr Val Lys Asp Pro Ala Arg Pro Trp Asn Val Leu Ala
100 105 110

Gln Glu Val Val Val Phe Glu Asp Gln Asp Ala Leu Leu Pro Cys Leu
115 120 125

Leu Thr Asp Pro Val Leu Glu Ala Gly Val Ser Leu Val Arg Val Arg
130 135 140

Gly Arg Pro Leu Met Arg His Thr Asn Tyr Ser Phe Ser Pro Trp His
145 150 155 160

Gly Phe Thr Ile His Arg Ala Lys Phe Ile Gln Ser Gln Asp Tyr Gln
165 170 175

Cys Ser Ala Leu Met Gly Gly Arg Lys Val Met Ser Ile Ser Ile Arg
180 185 190

Leu Lys Val Gln Lys Val Ile Pro Gly Pro Pro Ala Leu Thr Leu Val
195 200 205

Pro Ala Glu Leu Val Arg Ile Arg Gly Glu Ala Ala Gln Ile Val Cys
210 215 220

Ser Ala Ser Ser Val Asp Val Asn Phe Asp Val Phe Leu Gln His Asn
225 230 235 240

Asn Thr Lys Leu Ala Ile Pro Gln Gln Ser Asp Phe His Asn Asn Arg
245 250 255

Tyr Gln Lys Val Leu Thr Leu Asn Leu Asp Gln Val Asp Phe Gln His

260 265 270

Ala Gly Asn Tyr Ser Cys Val Ala Ser Asn Val Gln Gly Lys His Ser
275 280 285

Thr Ser Met Phe Phe Arg Val Val Glu Ser Ala Tyr Leu Asn Leu Ser
290 295 300

Ser Glu Gln Asn Leu Ile Gln Glu Val Thr Val Gly Glu Gly Leu Asn
305 310 315 320

Leu Lys Val Met Val Glu Ala Tyr Pro Gly Leu Gln Gly Phe Asn Trp
325 330 335

Thr Tyr Leu Gly Pro Phe Ser Asp His Gln Pro Glu Pro Lys Leu Ala
340 345 350

Asn Ala Thr Thr Lys Asp Thr Tyr Arg His Thr Phe Thr Leu Ser Leu
355 360 365

Pro Arg Leu Lys Pro Ser Glu Ala Gly Arg Tyr Ser Phe Leu Ala Arg
370 375 380

Asn Pro Gly Gly Trp Arg Ala Leu Thr Phe Glu Leu Thr Leu Arg Tyr
385 390 395 400

Pro Pro Glu Val Ser Val Ile Trp Thr Phe Ile Asn Gly Ser Gly Thr
405 410 415

Leu Leu Cys Ala Ala Ser Gly Tyr Pro Gln Pro Asn Val Thr Trp Leu
420 425 430

Gln Cys Ser Gly His Thr Asp Arg Cys Asp Glu Ala Gln Val Leu Gln
435 440 445

Val Trp Asp Asp Pro Tyr Pro Glu Val Leu Ser Gln Glu Pro Phe His
450 455 460

Lys Val Thr Val Gln Ser Leu Leu Thr Val Glu Thr Leu Glu His Asn
465 470 475 480

Gln Thr Tyr Glu Cys Arg Ala His Asn Ser Val Gly Ser Gly Ser Trp

485 490 495

Ala Phe Ile Pro Ile Ser Ala Gly Ala His Thr His Pro Pro Asp Glu
500 505 510

Phe Leu Phe Thr Pro Val Val Val Ala Cys Met Ser Ile Met Ala Leu
515 520 525

Leu Leu Leu Leu Leu Leu Leu Leu Leu Tyr Lys Tyr Lys Gln Lys Pro
530 535 540

Lys Tyr Gln Val Arg Trp Lys Ile Ile Glu Ser Tyr Glu Gly Asn Ser
545 550 555 560

Tyr Thr Phe Ile Asp Pro Thr Gln Leu Pro Tyr Asn Glu Lys Trp Glu
565 570 575

Phe Pro Arg Asn Asn Leu Gln Phe Gly Lys Thr Leu Gly Ala Gly Ala
580 585 590

Phe Gly Lys Val Val Glu Ala Thr Ala Phe Gly Leu Gly Lys Glu Asp
595 600 605

Ala Val Leu Lys Val Ala Val Lys Met Leu Lys Ser Thr Ala His Ala
610 615 620

Asp Glu Lys Glu Ala Leu Met Ser Glu Leu Lys Ile Met Ser His Leu
625 630 635 640

Gly Gln His Glu Asn Ile Val Asn Leu Leu Gly Ala Cys Thr His Gly
645 650 655

Gly Pro Val Leu Val Ile Thr Glu Tyr Cys Cys Tyr Gly Asp Leu Leu
660 665 670

Asn Phe Leu Arg Arg Lys Ala Glu Ala Met Leu Gly Pro Ser Leu Ser
675 680 685

Pro Gly Gln Asp Pro Glu Gly Gly Val Asp Tyr Lys Asn Ile His Leu
690 695 700

Glu Lys Lys Tyr Val Arg Arg Asp Ser Gly Phe Ser Ser Gln Gly Val

705 710 715 720

Asp Thr Tyr Val Glu Met Arg Pro Val Ser Thr Ser Ser Asn Asp Ser
725 730 735

Phe Ser Glu Gln Asp Leu Asp Lys Glu Asp Gly Arg Pro Leu Glu Leu
740 745 750

Arg Asp Leu Leu His Phe Ser Ser Gln Val Ala Gln Gly Met Ala Phe
755 760 765

Leu Ala Ser Lys Asn Cys Ile His Arg Asp Val Ala Ala Arg Asn Val
770 775 780

Leu Leu Thr Asn Gly His Val Ala Lys Ile Gly Asp Phe Gly Leu Ala
785 790 795 800

Arg Asp Ile Met Asn Asp Ser Asn Tyr Ile Val Lys Gly Asn Ala Arg
805 810 815

Leu Pro Val Lys Trp Met Ala Pro Glu Ser Ile Phe Asp Cys Val Tyr
820 825 830

Thr Val Gln Ser Asp Val Trp Ser Tyr Gly Ile Leu Leu Trp Glu Ile
835 840 845

Phe Ser Leu Gly Leu Asn Pro Tyr Pro Gly Ile Leu Val Asn Ser Lys
850 855 860

Phe Tyr Lys Leu Val Lys Asp Gly Tyr Gln Met Ala Gln Pro Ala Phe
865 870 875 880

Ala Pro Lys Asn Ile Tyr Ser Ile Met Gln Ala Cys Trp Ala Leu Glu
885 890 895

Pro Thr His Arg Pro Thr Phe Gln Gln Ile Cys Ser Phe Leu Gln Glu
900 905 910

Gln Ala Gln Glu Asp Arg Arg Glu Arg Asp Tyr Thr Asn Leu Pro Ser
915 920 925

Ser Ser Arg Ser Gly Gly Ser Gly Ser Ser Ser Ser Glu Leu Glu Glu

930 935 940

Glu Ser Ser Ser Glu His Leu Thr Cys Cys Glu Gln Gly Asp Ile Ala
945 950 955 960

Gln Pro Leu Leu Gln Pro Asn Asn Tyr Gln Phe Cys
965 970

<210> 11
<211> 1696
<212> DNA
<213> Homo sapiens

<400> 11
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<210> 12
<211> 466
<212> PRT
<213> Homo sapiens

<400> 12

Met Thr Pro Ile Leu Thr Val Leu Ile Cys Leu Gly Leu Ser Leu Gly
1 5 10 15

Pro Arg Thr His Val Gln Ala Gly His Leu Pro Lys Pro Thr Leu Trp
20 25 30

Ala Glu Pro Gly Ser Val Ile Ile Gln Gly Ser Pro Val Thr Leu Arg
35 40 45

Cys Gln Gly Ser Leu Gln Ala Glu Glu Tyr His Leu Tyr Arg Glu Asn
50 55 60

Lys Ser Ala Ser Trp Val Arg Arg Ile Gln Glu Pro Gly Lys Asn Gly
65 70 75 80

Gln Phe Pro Ile Pro Ser Ile Thr Trp Glu His Ala Gly Arg Tyr His
85 90 95

Cys Gln Tyr Tyr Ser His Asn His Ser Ser Glu Tyr Ser Asp Pro Leu
100 105 110

Glu Leu Val Val Thr Gly Ala Tyr Ser Lys Pro Thr Leu Ser Ala Leu
115 120 125

Pro Ser Pro Val Val Thr Leu Gly Gly Asn Val Thr Leu Gln Cys Val
130 135 140

Ser Gln Val Ala Phe Asp Gly Phe Ile Leu Cys Lys Glu Gly Glu Asp
145 150 155 160

Glu His Pro Gln Arg Leu Asn Ser His Ser His Ala Arg Gly Trp Ser
165 170 175

Trp Ala Ile Phe Ser Val Gly Pro Val Ser Pro Ser Arg Arg Trp Ser
180 185 190

Tyr Arg Cys Tyr Ala Tyr Asp Ser Asn Ser Pro Tyr Val Trp Ser Leu
195 200 205

Pro Ser Asp Leu Leu Glu Leu Leu Val Pro Gly Val Ser Lys Lys Pro
210 215 220

Ser Leu Ser Val Gln Pro Gly Pro Met Val Ala Pro Gly Glu Ser Leu
225 230 235 240

Thr Leu Gln Cys Val Ser Asp Val Gly Tyr Asp Arg Phe Val Leu Tyr
245 250 255

Lys Glu Gly Glu Arg Asp Phe Leu Gln Arg Pro Gly Trp Gln Pro Gln
260 265 270

Ala Gly Leu Ser Gln Ala Asn Phe Thr Leu Gly Pro Val Ser Pro Ser
275 280 285

His Gly Gly Gln Tyr Arg Cys Tyr Ser Ala His Asn Leu Ser Ser Glu
290 295 300

Trp Ser Ala Pro Ser Asp Pro Leu Asp Ile Leu Ile Thr Gly Gln Phe
305 310 315 320

Tyr Asp Arg Pro Ser Leu Ser Val Gln Pro Val Pro Thr Val Ala Pro
325 330 335

Gly Lys Asn Val Thr Leu Leu Cys Gln Ser Arg Gly Gln Phe His Thr
340 345 350

Phe Leu Leu Thr Lys Glu Gly Ala Gly His Pro Pro Leu His Leu Arg
355 360 365

Ser Glu His Gln Ala Gln Gln Asn Gln Ala Glu Phe Arg Met Gly Pro
370 375 380

Val Thr Ser Ala His Val Gly Thr Tyr Arg Cys Tyr Ser Ser Leu Ser
385 390 395 400

Ser Asn Pro Tyr Leu Leu Ser Leu Pro Ser Asp Pro Leu Glu Leu Val
405 410 415

Val Ser Ala Ser Leu Gly Gln His Pro Gln Asp Tyr Thr Val Glu Asn
420 425 430

Leu Ile Arg Met Gly Val Ala Gly Leu Val Leu Val Val Leu Gly Ile
435 440 445

Leu Leu Phe Glu Ala Gln His Ser Gln Arg Ser Leu Gln Asp Ala Ala
450 455 460

Gly Arg
465

<210> 13
<211> 63
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 13
ggccagtgaa ttgtaatacg actcactata gggaggcggt ttttttttt ttttttttt 60
ttt 63

<210> 14
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 14
gtcgtcaaga tgctaccgtt cagga 25

<210> 15
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 15
ggggacaagt ttgtacaaaa aagcaggcta tggaaaccaa cttctcca 48

<210> 16
<211> 53
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 16
ggggaccact ttgtacaaga aagctgggtt cacattgcct gtaactcagt ctc 53

<210> 17
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 17
agcccatagc agatggcaac 20

<210> 18
<211> 24
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer

<400> 18
tgtactttca actttgcatc ctgg 24

<210> 19
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 19
aagccaatga caaaccggat aatccctc 28

<210> 20
<211> 2051
<212> DNA
<213> Homo sapiens

<400> 20
cgccactttg ctggagcatt cactaggcga ggcgctccat cggactcact agccgcactc 60
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tccagccgga ttttcctgtt caacctggca gtagctgact ttctactgat catctgcctg 300
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attaaaaggg aaacgtgcct ctgccccacg ggtagagggg gtgcacgttc ctcctggttc 1980
cttcgcttgt gtttctgtac ttaccaaaaa tctaccactt caataaattt tgataggaga 2040
caaaaaaaaa a 2051

<210> 21
<211> 387
<212> PRT
<213> Homo sapiens

<400> 21

Met Asn Arg His His Leu Gln Asp His Phe Leu Glu Ile Asp Lys Lys
1 5 10 15

Asn Cys Cys Val Phe Arg Asp Asp Phe Ile Ala Lys Val Leu Pro Pro
20 25 30

Val Leu Gly Leu Glu Phe Ile Phe Gly Leu Leu Gly Asn Gly Leu Ala
35 40 45

Leu Trp Ile Phe Cys Phe His Leu Lys Ser Trp Lys Ser Ser Arg Ile
50 55 60

Phe Leu Phe Asn Leu Ala Val Ala Asp Phe Leu Leu Ile Ile Cys Leu
65 70 75 80

Pro Phe Val Met Asp Tyr Tyr Val Arg Arg Ser Asp Trp Asn Phe Gly
85 90 95

Asp Ile Pro Cys Arg Leu Val Leu Phe Met Phe Ala Met Asn Arg Gln
100 105 110

Gly Ser Ile Ile Phe Leu Thr Val Val Ala Val Asp Arg Tyr Phe Arg
115 120 125

Val Val His Pro His His Ala Leu Asn Lys Ile Ser Asn Trp Thr Ala
130 135 140

Ala Ile Ile Ser Cys Leu Leu Trp Gly Ile Thr Val Gly Leu Thr Val
145 150 155 160

His Leu Leu Lys Lys Lys Leu Leu Ile Gln Asn Gly Pro Ala Asn Val
165 170 175

Cys Ile Ser Phe Ser Ile Cys His Thr Phe Arg Trp His Glu Ala Met
180 185 190

Phe Leu Leu Glu Phe Leu Leu Pro Leu Gly Ile Ile Leu Phe Cys Ser
195 200 205

Ala Arg Ile Ile Trp Ser Leu Arg Gln Arg Gln Met Asp Arg His Ala
210 215 220

Lys Ile Lys Arg Ala Ile Thr Phe Ile Met Val Val Ala Ile Val Phe
225 230 235 240

Val Ile Cys Phe Leu Pro Ser Val Val Val Arg Ile Arg Ile Phe Trp
245 250 255

Leu Leu His Thr Ser Gly Thr Gln Asn Cys Glu Val Tyr Arg Ser Val
260 265 270

Asp Leu Ala Phe Phe Ile Thr Leu Ser Phe Thr Tyr Met Asn Ser Met
275 280 285

Leu Asp Pro Val Val Tyr Tyr Phe Ser Ser Pro Ser Phe Pro Asn Phe
290 295 300

Phe Ser Thr Leu Ile Asn Arg Cys Leu Gln Arg Lys Met Thr Gly Glu
305 310 315 320

Pro Asp Asn Asn Arg Ser Thr Ser Val Glu Leu Thr Gly Asp Pro Asn
325 330 335

Lys Thr Arg Gly Ala Pro Glu Ala Leu Met Ala Asn Ser Gly Glu Pro
340 345 350

Trp Ser Pro Ser Tyr Leu Gly Pro Thr Ser Asn Asn His Ser Lys Lys
355 360 365

Gly His Cys His Gln Glu Pro Ala Ser Leu Glu Lys Gln Leu Gly Cys
370 375 380

Cys Ile Glu
385